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(54) Title: ENZYME STABILISATION

(57) Abstract

A method of stabilising enzymes or other proteins against denaturation or drying comprises mixing the protein with a cyclic polyol and a cationic polyelectrolyte.

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ENZYME STABILISATION

This invention relates to stabilisation of proteins, particularly but not exclusively of enzymes in the dry state.

Few enzymes are inherently stable in solution. Many have a tendency to become denatured when held in solution. Various workers have attempted to stabilise enzymes either by adding compounds such as sugars or glycerol to solutions of them or by freeze drying. These methods often cause a loss of activity. Alternative methods of stabilisation have involved drying of enzymes with stabilisers in a presence of a solid support such as cellulose fibre or polyacrylamide. US 4451569 disclosed stabilisation of glutathione peroxidase by freezing the enzyme with one of a number of sugars including arabinose, glucose, xylitol and sorbitol. Freeze drying is expensive to operate on a large scale and often results in denaturation.

PCT/GB86/00396 discloses stabilisation of proteins by use of the disaccharide trehalose.

According to a first aspect of the present invention a method of protecting proteins against denaturation on drying comprises mixing an aqueous solution of the protein with a soluble cationic polyelectrolyte and a cyclic polyol, and removing water from the solution.

Stabilisation in accordance with this invention enhances the activity of freshly dried enzymes and other proteins. The stability upon storage is also enhanced.

The proteins may include enzymes, antibodies, antigens, serum complement, vaccine components and bioactive peptides.

Drying of proteins and especially enzymes is important for many applications, for example use in diagnostic or analytical aids such as test strips which may be stored for prolonged periods before use. Transportation of enzymes or other proteins in solution is inconvenient and expensive.

Although freeze drying may be employed, the present invention facilitates use of the vacuum drying and air drying

without denaturation. Vacuum drying and air drying milder processes and are much cheaper to operate.

The cyclic polyol may incoporate one or more alicyclic rings and may have at least one side chain. Compounds having 5 to 10 hydroxyl groups may be preferred. Non-reducing polyols are preferred. Di and trisaccharides are particularly efficaceous but other cyclic polyols, for example inositol may also be used. The polyol may be chosen to suit both the enzyme or other protein and also the polyelectrolyte in question. Lactitol, lactose, maltose and sucrose are especially preferred in conjuction with DEAE-dextran, lactitol having been found to be most suitable for many applications. Sorbitol is suitable for use with cholesterol oxidase, cholesterol esterase and other enzymes. Cellobiose may also be used. The amount of polyol may lie in the preferred range of 1 to 20%, more preferably 2 to 10%, most preferably 5 to 10%.

The cationic polyelectrolyte is preferably a polymer with cationic groups distributed along the molecular chain. The cationic groups, which are preferably quaternary ammonium derived functions, may be disposed in side groups pendent from the chain or may be incorporated in it. Natural or artificial polymers may be employed. Natural polymers such as polysaccharides are preferred since many artificial polymers contain residual traces of the inorganic polymerisation catalyst.

Diethylaminoethyl dextran (DEAE-dextran) and chitosan are preferred although polyethyleneimine is also suitable. Polysaccharides with MW 5000 to 500 000, preferably 5000 to 20 000, more preferably 5000 to 10 000 may be employed. An amount of 0.1 to 10% is preferred, especially 0.5 to 2%.

The pH at which enzymes are dried in accordance with this invention may be important to optimise retention of activity both upon drying and after subsequent storage. The optimum pH for a particular enzyme may be determined by simple experimentation.

Alcohol oxidase has been formed to retain acivity between pH 7 and 8, preferably at pH 7.8.

Cholesterol oxidase, dependent on the source, dries best at

pH 5 or 9.

Uricase may be dried at pH 9.

Cholesterol esterase dependent on source may be dried at pH 7 or 9.

Drying is preferably performed in the presence of a wetting agent. Temperatures between 4° and 50°, especially 25° to 35° are preferred.

According to a second aspect of the present invention there is provided a dried product containing a protein, cyclic polyol and cationic polyelectrolyte.

The dried product may be a free running powder or may comprise part of a test strip or other analytical or diagnostic apparatus.

The present invention is now described by means of example but not in any limitative sense.

Experimental Procedures

Percentages used in the specification are by weight unless indicated otherwise.

All the stabilisation systems utilise buffers to maintain stable pH conditions eg.

Buffer solutions containing $Na_2HPO_4.2H_2O$ (10.855g) and $NaH_2PO_4.2H_2O$ (6.084g) were dissolved in 1.0 litre distilled water to give a solution of pH 7.0 at a concentration of 100 millimoles per litre.

An alternative buffer is MOPS - (4-Morpholine Propane Sulphuric Acid) -52.25g/2.5l distilled water pH to 7.87 with 4.0M.NaOH.

A wetting agent may be used dependent on whether or not the enzyme system is being stabilised in a polystyrene cuvette. A suitable wetting agent is protein hydrolysate from gelatine termed Byco A These are made up to 1% w/v in phosphate buffer, 100 $mmol.l^{-1}$, pH 7.0 as needed.

Enzyme solutions were made up freshly before use. Stock solutions of enzymes in ammonium sulphate solution were dialysed

exhaustively against buffer eg 100 $mmol.l^{-1}$ phosphate buffer pH 7.0 to remove all salts.

Stock enzyme concentrations may be from 10 to 1000 units of activity per millilitre of solution. In terms of protein concentration this is between 0.5 to 200 $mgcm^{-3}$. Typically the final protein concentration was 1.0 $mgcm^{-3}$.

Soluble polyelectrolytes, polyols , enzyme, buffer salts and wetting agent (if used) were mixed at constant temperature and dried in a vacuum oven over dessicant eg. silica gel, 0.1mm/Hg, 30°C for 4-10 hr.

The oxidase enzymes studied may be assayed by colorimetric detection of the hydrogen peroxide produced by action of the enzyme. Peroxidase acts on the hydrogen peroxide produced in the presence of aromatic alcohols or amines and the heterocyclic compound 4-aminoantipyrine to give quinoneimine dyes. Other standard assays systems may be employed eg u.v. spectrometry.

The following systems were employed:

System 1

Phenol sulphonic acid	25 $mmol.l^{-1}$
4-aminoantipyrine	0.4 $mmol.l^{-1}$
Peroxidase	1000 unit/l

The resultant dye was measured at 500nm.

System 2

3,5-dichloro 2-hydroxybenzene sulphonic acid	10 $mmol.l^{-1}$
4-aminoantipyrine	0.4 $mmol.l^{-1}$
Peroxidase	1000 units/l

The resultant dye was measured at 520 nm.

Standard temperature eg. 25°C and incubation times eg. 5

minutes were used. Reagent blanks contained all components except substrate. Dry preparations in cuvettes were reconstituted with Systems 1. or 2. directly.

Dry powdered preparations were reconstituted with phosphate buffer and suitable aliquots were added to System 1. or 2.

For stability trials the storage temperature was 37°C, with samples being removed periodically to check for residual activity of the enzyme. This procedure was standard for all enzymes tested.

Soluble Polyelectrolyte and Sugar Alcohol or Saccharide

Soluble Polyelectrolyte

Soluble polyelectrolyte was dissolved in distilled water a concentration up to 20% w/v, usually to 10% w/v. Sugar alcohol or saccharide was dissolved in distilled water up to a concentration of 40% w/v, usually to 20% w/v. These solutions were used within 4 weeks of preparation, being stored in the cold at 4°C.

Example 1

Solution 1	DEAE-Dextran	10%	100ul
	Lactitol	20%	500ul
	Byco A	1%	100ul
Solution 2	Alcohol oxidase 7 units (1.7mg protein		35ul
	Phosphate buffer $100mmol.l^{-1}$ pH 7.0		265ul

Solution 1 was stirred continuously whilst slowly adding Solution 2 at 4°C. The mixture was stirred for 5 minutes to ensure complete mixing. 0.1ml volumes were dried in cuvettes as described, stored at 37°C and assayed for activity as described

(Table 1).

Example 2

Solution 1	Alcohol oxidase 2 411 units (=422 mg protein) in phosphate buffer 300mmol.l^{-1}	2.7cm^3
Solution 2	Lactitol 20% w/v	3.0cm^3
	DEAE-Dextran 10% w/v	0.27cm^3

Solution 2 was added slowly to Solution 1 with stirring. The mixed solutions were pipetted into petri dishes and vacuum dried over silica gel at 30°C for 8 hours whereupon a thin glassy film of dried enzyme and stabiliser was produced. This was removed and ground to a fine powder using a glass pestle and mortar.

For stability testing 10mg portions of enzyme powder were weighed into sterile polystyrene tubes and incubated at 37° in a sealed container over silica gel. Samples were removed periodically and reconstituted in distilled water. 60ul of reconstituted enzyme solution was added to each assay cuvette containing peroxidase and colour reagents as described (Table 2).

Example 3

Solution 1	DEAE-Dextran	10% w/v	100ul
	Lactitol	20% w/v	500ul
	Byco A	1% w/v	100ul
Solution 2	Choline oxidase 10 units (0.794 mg protein) in phosphate pH 7.0 100cm^3		300ul

Solution 2 was added with stirring to Solution 1 and thoroughly mixed at 4°C. 0.1cm^3 volumes were vacuum dried in

cuvettes as described, stored at 37°C and assayed for activity as described (Table 3).

Example 4

Glycerol 3 Phosphate Oxidase

Solution 1	DEAE-Dextran	10% w/v	100ul
	Lactitol	20% w/v	500ul
	Byco A	1% w/v	100ul
Solution 2	Glycerol 3 Phosphate Oxidase 10 units (0.526 mg protein) in phosphate buffer pH 7.0 100mmol.l^{-1}		300ul

Solution 2 was added with stirring to Solution 1 at 4°C and thoroughly mixed. 0.1cm^3 volumes were vacuum dried in cuvettes as described (Table 4).

Example 5

Solution 1	DEAE-Dextran	10%	100ul
	Lactose	20%	500ul
	Byco A	1%	100ul
Solution 2	Alcohol oxidase 5 units (1.0mg protein in 100mmol.l^{-1} Phosphate buffer pH 7.0		300ul

Solution 1 was stirred continuously whilst slowly adding Solution 2 at 4°C. The mixture being stirred for 5 minutes to ensure complete mixing 0.1cm^3 volumes were dried in cuvettes as described (Table 5).

Soluble Polysaccharides

Soluble polysaccharides were dissolved in distilled water up to a concentration of 30% w/v usually to a concentration of 10% w/v. These solutions were used within 4 weeks of preparation and stored at 4°C.

Example 6

Solution 1	Dextran (molecular wt. 10 000) 10% w/v	100ul
	Byco A 1% w/v	100ul
	Distilled water	500ul
Solution 2	Alcohol oxidase 7 units (1.32 mg protein) in 100mmol.l^{-1} Phosphate buffer pH 7.0	300ul

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm^3 volumes were vacuum dried in cuvettes, stored at 37°C and assayed for activity as described.

When dextrans of differing molecular weights are used variations in stability were noted (Table 6).

Exmaple 7

Solution 1	Dextran molecular wt. 10 000 10% w/v solution	500ul
	Phosphate buffer 10mmol.l^{-1} pH 7.0	300ul
Solution 2	Galactose oxidase 0.52 units (0.8 mg protein) in 10mmol.l^{-1} phosphate buffer pH 7.0	200ul

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing.

0.1 ml aliquots were vacuum dried, stored at 37°C and assayed for activity as described (Table 7).

Cyclic Polyalcohol

Cyclic polyalcohol was dissolved in distilled water to a concentration of 10% w/v. The solutions were stored at 4°C and used within 4 weeks of preparation.

Example 8

Solution 1	Inositol 10% w/v	500ul
	Distilled water	200ul
Solution 2	Alcohol oxidase 4.7 units 91.15mg/protein) in Phosphate buffer 100mmol.l^{-1}	200ul

Solution 2 was added to Solution 1 with stirring t 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm^3 aliquots were vacuum dried, stored at 37°C and assayed for activity as described (Table 8).

Example 9

Solution 1	Inositol 10% w/v	500ul
	Phosphate buffer 100mmol.l^{-1} pH 7.0	300ul
Solution 2	Galactose oxidase 0.52 units (0.8mg protein) in 10mmol.l^{-1} phosphate buffer pH 7.0	200ul

Solution 2 was added to Solution 1 with stirring at 4°C and stirring was continued for 5 minutes to ensure complete mixing. 0.1cm^3 aliquots were vacuum dried as described, stored at 37°C

and assayed for activity as described (Table 9).

Example 10

The following results show the stabilisation of alcohol oxidase (Hansenula polymorpha).

Unstabilised enzyme retained 26% activity after 7 days incubation at 37°C. Addition of chitosan above gave retention of 48.9% activiy after 9 days. The activity in relation to freshly dried enzyme was measured after incubation at 37°C.

Stabiliser	Period of incubation /days	Activity/%
lactitol 5% chitosan 0.1%	1	86.9
	6	85.7
	9	82.1
	16	86.1
lactitol 5% chitosan 0.01%	1	87.4
	6	87.2
	9	83.4
	16	91.6
lactitol 5% polyethyleneimine 0.1%	1	79.3
	6	77.5
	9	76.1
	16	77.5

lactitol 5%	1	91.1
Polyethyleneimine 0.01%	6	84.4
	9	96.1
	16	93.1
lactitol 5%	1	94.9
DEAE-Dextran 0.1%	6	85.1
	9	88.7
	16	90.6
lactitol 5%	1	98.3
DEAE-Dextran 0.01%	6	88.8
	9	89.4
	16	95.9

Example 11

The following results show stabilisation of alcohol oxidase (Pichia pastoris). Unstabilised enzyme retained 49.8% and 36.1% activities after 2 days and 13 days respectively at 37°C. Enhanced activity (ie greater than 1-%) upon drying may be attributable to selective degradation of inhibiting impurities.

Stabiliser	Period of incubation days	Activity/%
lactitol 5%	1	102.5
DEAE-Dextran 1%	4	116.6

	8	121.3
	15	104.3
dextran 5%	1	83.2
	4	97.0
	8	101.7
	15	87.6
inositol 5%	1	88.0
	4	106.2
	8	107.1
	15	109.1

Example 12

The following results illustrate stabilisation of cholesterol oxidase (Nocardia erythropolis). Unstabilised enzyme retained 34.3% activity after 14 days at 37°C.

Stabiliser	Period of incubation days	Activity/%
lactitol 5%	3	96.2
DEAE-dextran 1%	5	105.6
	14	115.7
inositol 5%	1	92.6
	7	84.8
	10	91.7

Example 13

The following results illustrate stabilisation of freeze dried uricase.

Stabiliser	Period of incubation days	Activity/%
lactitol 5% DEAE-dextran 1%	1	109.9
	5	114.3
	10	109.9

Example 14

The following results illustrate stabilisation of various enzymes with lactitol (15%) and DEAE-dextran (1%) during drying in comprarison to the activity of undried enzymes.

Enzyme	Activity after drying/%	
	Unstabilised	Stabilised
Alcohol oxidase (Pichia)	64.7	78.2
Choline oxidase	63.3	97.7
Lactate oxidase	77.1	90.0
Alcohol oxidase (Hensenula polymorpha)	68.2	119.6
Cholesterol oxidase (vacuum dried)	80.0	92.5 (inositol 5%)
(freeze dried)	79.0	91.0 (inositol 5%)

TABLE 1

Preparation	Incubation 37°C	% Activity remaining relative to activity freshly dried enzyme
Alcohol oxidase + Lactitol 10% DEAE-Dextran 1%	1 day	108
	7 days	120
	14 days	114
	21 days	106
	5 months	102

Unstabilised Enzyme retained 26% activity after 7 days

TABLE 2

Bulk preparation	Incubation 37°C	% Activity retained relative to freshly dried enzyme
DEAE-Dextran 1% Lactitol 10%	4 days	138
Alcohol oxidase 7.25 units/10mg solid	12 days	121

Unstabilised Enzyme retained 34% activity after 4 days.

TABLE 3

Preparation	Incubation 37°C	% Activity remaining relative to freshly dried enzyme
Choline oxidase	1 day	99
DEAE-Dextran 1%	5 days	84
Lactitol 10%	10 days	81
	15 days	83

Unstabilised Enzyme retained 24% of activity after 1 day, decreasing to 11% after 5 days.

TABLE 4

Preparation	Incubation 37°C	% Activity remaining relative to freshly dried enzyme
Glycerol 3 phosphate oxidase	1 days	104
	5 days	120
DEAE-Dextran 1%	10 days	117
Lactitol 10%	15 days	113

Unstabilised enzyme retained 94% activity after 1 day but only retained 54% activity after 15 days.

TABLE 5

Preparation	Incubation 37°C	% Activity remaining relative to freshly dried enzyme
Alcohol oxidase	1 day	137
DEAE-Dextran 1% wt/vol	6 days	103
Lactose 10% wt/vol	10 days	108
	15 days	92

Unstabilised enzyme retained 23% of activity after 10 days at 37°C.

TABLE 6

Preparation	% Activity remaining (relative to freshly dried enzyme)			
Alcohol oxidase + Dextran 1% wt/vol	1 day 37°C	6 days 37°C	18 days 37°C	11 months 37°C
M Wt.				
T10 10 000	93	87	73	77
T40 40 000	82	75	61	64
T70 70 000	84	86	65	60
T500 500 000	83	86	62	49
T2000 2 000 000	45	43	22	14

Unstabilised enzyme retained 30% of activity after 6 days.

TABLE 7

Galactose oxidase

Dextran concentrations 5% (M.W 10 000).

Incubation 37°C	% Activity remaining relative to activity of freshly dried enzyme.
1 day	92%
7 days	87%
10 days	82%

TABLE 8

Preparation	Incubation 37°C	% Activity remaining relative to freshly dried enzyme
Alcohol oxidase + 5% Inositol	1 Day	150%
	7 days	196%
	14 days	166%
	23 days	178%

Unstabilised enzyme retained 26% activity after 7 days.

TABLE 9

Preparation	Incubation 37°C	% Activity remaining relative to freshly dried enzyme
Galactose oxidase + 5% Inositol	1 day	85%
	4 days	78%
	10 days	74%

CLAIMS

1. A method of protecting proteins against denaturation on drying comprising the steps of:

mixing an aqeuous solution of the protein with a soluble cationic polyelectrolyte and a cyclic polyol, and

removing water from the solution.

2. A method as claimed in Claim 1, wherein said polyelectolyte comprises a quaternary ammonium functionalised polysaccharide.

3. A method as claimed in Claim 2, wherein said polyelectrolyte comprises diethylamminoethyl-dextran or chitosan.

4. A method as claimed in Claim 1, wherein the polyelectrolyte comprises polyethyleneimine.

5. A method as claimed in any preceding claim, wherein the polyol is a di or trisaccaride.

6. A method as claimed in Claim 5 wherein the polyol is selected from the group comprising: lactitol, lactose, maltose sucrose and cellobiose.

7. A method as claimed in any preceding claim, wherein water is removed at a temperature between 4° and 50°C.

8. A method as claimed in Claim 7, wherein the temperature is 25° to 35°C.

9. A dried product containing a protein, cyclic polyol and a cationi polyelectrolyte.

10. A dried product as claimed in Claim 9 containing an enzyme, cyclic polyol and quaternary ammionium functionalised polymeric polysaccharide.

11. A dried product as claimed in Claim 10, containing an enzyme, cyclic polyol and a polyethyleneimine.

12. A dried product as claimed in any of Claims 9 to 11, wherein the polyol is a di or trisaccharide.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01346

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 9/96

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Chemical Abstracts, vol. 89, no. 16, 16 October 1978, (Columbus, Ohio, US), see page 404, abstract 135880v & SU, A, 615933 (LENINGRAD SCIENTIFIC-RESEARCH INSTITUTE OF HEMATOLOGY AND BLOOD TRANSFUSION) 25 July 1978 --	1
A	FR, A, 2209776 (AJINOMOTO) 5 July 1974, see the whole document --	1
A	EP, A, 0136996 (MONSANTO CO.) 10 April 1985, see the whole document	4

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th February 1990	29.03.90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8901346 SA 32768

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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